

Gonadal recrudescence and induced spawning in *Barbus altianalis*

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Abstract

Gonadal recrudescence in *Barbus altianalis* from River Nile, Jinja (33°05E; 0°45N) Uganda was studied prior to induced spawning. Oocytes in *B. altianalis* were found to develop in cohorts suggesting batch spawning throughout the year with pronounced spawning activities coinciding with rainfall peaks of April and September. There was a strong positive correlation between rainfall and gonadosomatic index ($r_s = 0.75$, $P = 0.008$). *B. altianalis* had low fecundity (6.0 ± 2.0 per gram) and large-sized eggs (2.97 ± 0.1 mm). Induced spawning was successful with exogenous hormones and water current. Results from this study suggested that total striping may not be appropriate, but larger eggs would confer high survival of offsprings under optimal conditions. The success registered in induced spawning of *B. altianalis* provides a breakthrough in seed production for multiplication and culture of this economically important fish species.

Keywords: *Barbus altianalis*, gonado recrudescence, induced spawning

Introduction

Barbus altianalis locally known in Uganda as *Kisinja* is a large freshwater cyprinid fish whose population is threatened by overfishing and climate change. The species occurs naturally in many water bodies within the Lake Victoria basin and effluent rivers (Greenwood 1966). The species is migratory and often exploits lacustrine–riverine interconnectivity to spawn and grow (Tomasson,

Cambray & Jackson 1984; De Stefano & De Graaf 2003). *B. altianalis* is an omnivorous fish whose diet is associated with detritus dominated by algae, plant material, molluscs, chironomids and cardis fly larvae (Balirwa 1979). The fish is relished as food in East Africa and was once a significant component of the Lake Victoria fisheries before its populations drastically decreased (Witte, Gouwdswaard, Katunzi, Mkumbo, Seehausen & Wanink 1999). The decline of the species natural stocks has also been attributed to ecological changes that have taken place in Lake Victoria in addition to intense fishing pressure (Ogutu-Ohwayo 1990). *B. altianalis* grows in the wild to attain a size of 90-cm TL (Greenwood 1966) and closely resembles other cyprinids whose culture is prominent in Asia and the Pacific regions. Although cyprinid species are some of the most cultured group of fish in the world (Food & Agriculture Organization (FAO) 2008), only the common carp *Cyprinus carpio* is cultured to a large extent in Africa in spite of an array of indigenous cyprinids in lakes and rivers across the continent. The low level of cyprinid culture on the continent is associated with paucity of information on their reproductive biology to guide induced spawning and subsequent seed production. Understanding natural reproduction of species followed by induced spawning at the right stage of gonadal recrudescence has led to successful induced spawning of another cyprinid fish, *Labeo victorinus* (Rutaisire & Booth 2004) with a closely related natural distribution. This study investigated some aspects of the reproductive biology of *B. altianalis* and used the generated information to induce ovulation, fertilization, incubation and larval production.

Materials and methods

Collection of samples

A total of 210 female fish were collected monthly from the River Nile Kiira landing site, 33°05'E, 0°45'N (Fig. 1) between January and December 2008. A fleet of 10 horizontally joined single panels gillnets, each 40 m long, 2 m deep with 5-mm stretched mesh were set for 12 h at night. After capture, the fork length (FL) and standard length (SL) were measured to the nearest 0.1 mm and eviscerated body weight (W) to the nearest 0.01 g. The specimens were dissected to expose the viscera. The mass of the excised ovary was recorded and gonadosomatic index (GSI) calculated. The ovaries were preserved in Bouin's solution for histological analysis.

Extraction of estradiol (E₂)

About 3 mL of blood was collected from the caudal vein of each of the 95 fish samples using a

heparinized syringe. The samples were collected monthly for 12 months. The blood was allowed to stand on ice for 2 h and centrifuged at 3200 *g* for 15 min in Eppendorf tubes. Collected plasma was transferred into 1.5-mL vials, stored in liquid nitrogen tank (−196°C) and transferred to the laboratory of the Department of Animal Science at the Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem in Israel for analysis. In the laboratory, plasma steroid hormone E₂ was extracted with diethyl ether. Briefly, 300 µL of plasma was transferred to clean tubes and diethyl ether (5 mL) was added, vortexed three times for 10 s to solubilize the steroid in the organic phase, while the proteins remain in the aqueous phase. The mixture was transferred to −80°C for 30 min to allow separation of the ether and aqueous phases. The supernatant, consisting of steroids that dissolved in ether was quickly decanted into another tube and slowly evaporated at 55°C for 1 h. The steroids were then reconstituted in 0.3 mL of steroid assay

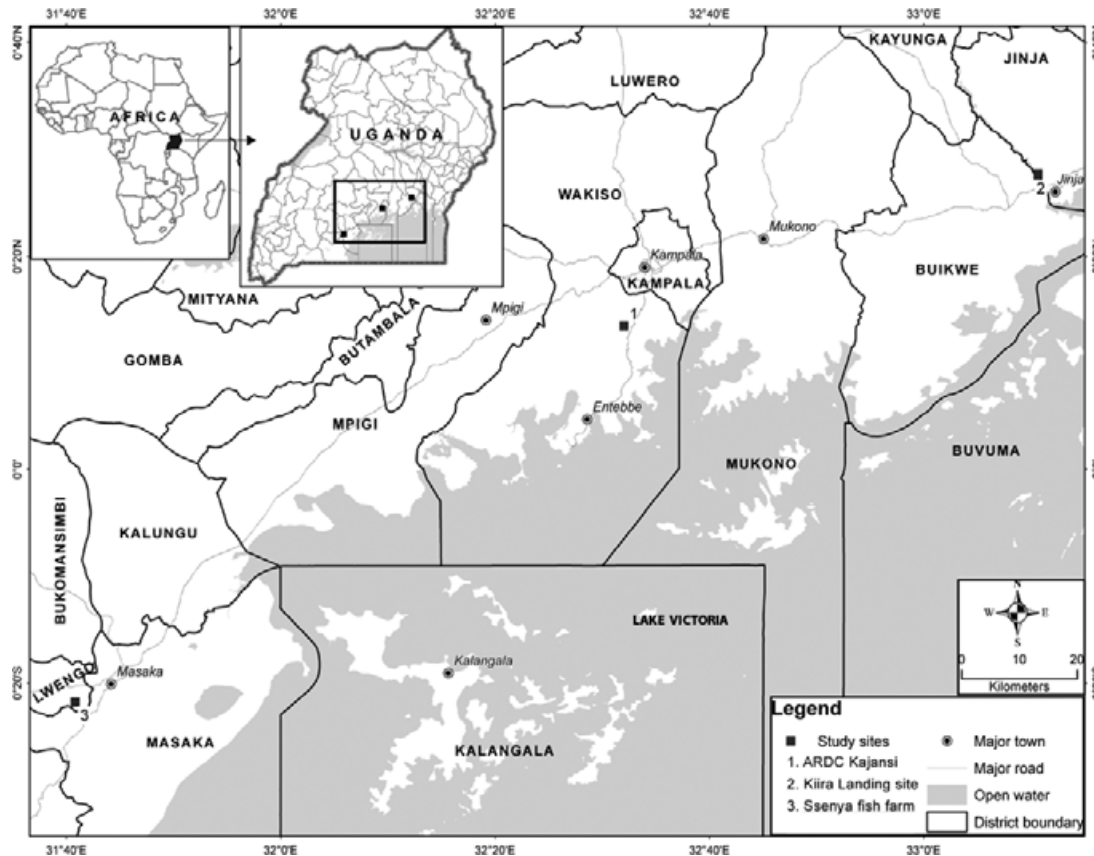


Figure 1 Map showing location of study site at Kiira Landing site, Ssenya Fish Farm and Kajansi Aquaculture Research and Development Center (ARDC).

buffer, vortexed for 30 s and transferred to -20°C in Eppendorf tubes until used in steroid enzyme-linked immunosorbent assay (ELISA).

The steroid concentration in the samples was determined using a specific competitive ELISA according to Levavi-Sivan, Vaiman, Sachs and Tzchori (2004) and Hurvitz, Degani, Goldberg, Yom-Din, Jackson and Levavi-Sivan (2005). All samples were analysed in duplicates, and a separate standard curve was run for each ELISA plate. Steroid levels in each sample medium determined using ELISA were validated by verifying that serial dilutions were parallel to the relevant standard curve. The lower limit of detection was 0.93 pg mL^{-1} ; the intra- and inter-assay coefficients of variance were less than 7% and 11% respectively.

Histological processing

Small tissue subsections were taken from rostral, middle and caudal regions of the preserved ovary and fixed in Bouin's fluid for 24 h prior to transfer to 70% ethanol for further histological processing at Makerere University, Faculty of Veterinary Medicine, Kampala Uganda. The samples were processed using standard histological methods (Bancroft & Gamble 2002). Processed ovarian sections were examined under a light microscope followed by oocyte staging and counting.

Fecundity

Fecundity was determined using gravimetric method (Bagenal 1978) on 60 females collected during the peak GSI months of April and September. Gonadal samples that were preserved in ethanol were removed and sectioned into rostra, caudal and middle portions. A mean sample was obtained and the total number of eggs estimated from the weight of both ovaries. Only the big uniform eggs ($2.4 \pm 0.5\text{ mm}$) that constituted a batch were counted collection of ripe brood fish for inducing to spawning.

Sexually mature *B. altianalis* were collected at a site where the Nile River exits Lake Victoria ($33^{\circ}05\text{E}$, $0^{\circ}45\text{N}$) on its course to the Mediterranean Sea. The first successful collection of brood stock and induced spawning was conducted in April 2008. Twelve ripe males and 15 ripe females, with average weight of 2.5 kg, were captured by gillnetting and transported to Aquaculture Research and Development Centre (ARDC)

at Kajjansi, in an aerated 500-litre PVC tank. On arrival, the fish were allowed to rest for 6 h in concrete tanks ($2 \times 4 \times 2\text{ m}$) prior to induction. During the acclimation, water flow into the tanks was maintained at 60 L min^{-1} while oxygen was delivered from a cylinder to maintain dissolved oxygen levels at above 4 mg L^{-1} . After 12 h, 10 females were found to have been induced to ovulate by the water current alone. These individuals were immediately stripped and fertilized with milt that was stripped from five males. Some fish from the flow tanks, however, did not ovulate. The fish that had not ovulated were weighed and given an intramuscular injection of the inducing agent DAGIN, [(D-Arg6, Pro9-NEt)]-sGnRH; $10\text{ }\mu\text{g kg}^{-1}$) combined with the dopamine receptor antagonist metoclopramide (GnRH + MET; 2 mg kg^{-1}) (Kibbutz Gan Shmuel Fish Breeding Centre, Israel; Drori, Ofir, Levavi-Sivan & Yaron 1994). Ovulation occurred 12 h after injecting the inducing agent and was confirmed by exudation of the oocytes upon application of slight pressure on the abdomen. Fertilization was through the dry method as described by Rothbard (1981). In subsequent trials, females in tanks without water current did not ovulate.

The eggs were spread onto plastic mesh trays ($0.75 \times 0.3\text{ m}$, with 0.6-mm mesh size) held in wooden frames. The incubation and hatching process was conducted in ARDC indoor hatchery with regulated environmental conditions, fixed at: temperature of $27 \pm 1^{\circ}\text{C}$ and dissolved oxygen $6 \pm 1\text{ mg L}^{-1}$, monitored using multi-parameter water quality test meter, model YSI- 550A. Fertilized eggs were counted, and the fertilization rate was determined. The time at the onset and at the end of hatching was recorded. Eggs that did not hatch were counted at the end of the incubation period. Egg size was determined by measuring the diameter of fertilized and unfertilized eggs, during egg incubation. Hatching success was defined as the percentage difference between unhatched and the total fertilized eggs at the start of incubation. Induced spawning experiments were repeated four times at ARDC and at Ssenya fish farm in Masaka (Fig. 1) during the rainy seasons of September to December 2009 and 2010. Fungal infections were controlled and prevented by the application of potassium permanganate bath at 0.02 g L^{-1} and 0.002 g L^{-1} both for 15 min and observation of maximum environmental hygiene.

Statistical analyses

The relationship between mean monthly gonadosomatic index (GSI) and mean monthly rainfall was investigated using Spearman's Rank Order correlation analysis because preliminary analyses indicated violation of the assumption of normality. A repeated measures ANOVA was used to determine if GSI and hormonal levels varied significantly among months. The Greenhouse–Geisser correction was used to adjust the degrees of freedom to correct for the violation of Sphericity assumption. Pair-wise multiple comparisons were done using the Bonferroni *Post hoc* tests. The relationship between fecundity and female body size (length and weight) was determined using simple linear regression analysis. All statistical analyses were performed using SPSS for Windows (Version 16; SPSS Inc., Chicago, IL, USA) at P value = 0.05.

Results

Reproductive seasonality

The maximum observed female GSI were recorded in April (3.46 ± 1.041) and September (4.125 ± 1.2789), whereas the minimum GSI occurred in February (1.725 ± 0.295). There was a significant positive correlation between rainfall and GSI ($r_s = 0.72$, $n = 12$, $P = 0.008$) with peaks in GSI occurring between April and May and between September and October, which coincided with rainfall peaks (Fig. 2). However, a repeated measures ANOVA indicated that mean GSI did not differ significantly among months ($P > 0.05$).

Estradiol variations

There were no significant differences in Estradiol levels among months ($P > 0.05$). However, variations among months showed a slight peak in April and a more pronounced one from September to October (Fig. 3).

Histological description of ovarian recrudescence

The process of oogenesis was classified according to oocyte location and size, staining characteristics, number of nucleoli, the presence and shape of the follicular layer, and the distribution of cytoplasm inclusions. According to these criteria, oogenesis was found to proceed through six stages namely: oogonia, chromatin nucleolus, perinuclear oocytes, cortical alveoli oocytes, secondary yolk vesicle oocytes and tertiary yolk vesicle oocytes (Fig. 4). Histological sections showed that oocytes in *B. altianalis* developed in distinct groups (Fig. 4H). All oocyte stages were present in the ovary throughout the annual cycle with the highest incidence of fresh post-ovulatory follicles observed in April and September (Figs 4 and 5).

Fecundity

The average number of eggs per gram was found to be 6 ± 3 eggs g^{-1} . Although there was a linear relationship between fecundity and fish size (Figs 6 and 7), no strong correlation was observed (length, $R^2 = 0.346$, $df = 53$, $P < 0.001$; weight, $R^2 = 0.328$, $df = 53$, $P < 0.001$).

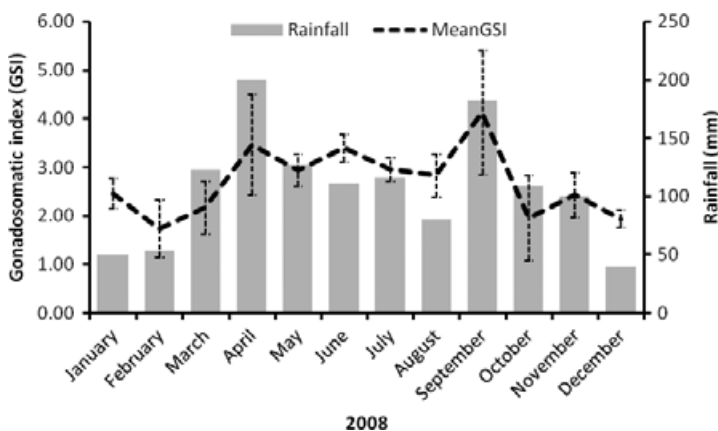


Figure 2 Monthly gonadosomatic index GSI of *Barbus altianalis* variation with rainfall.

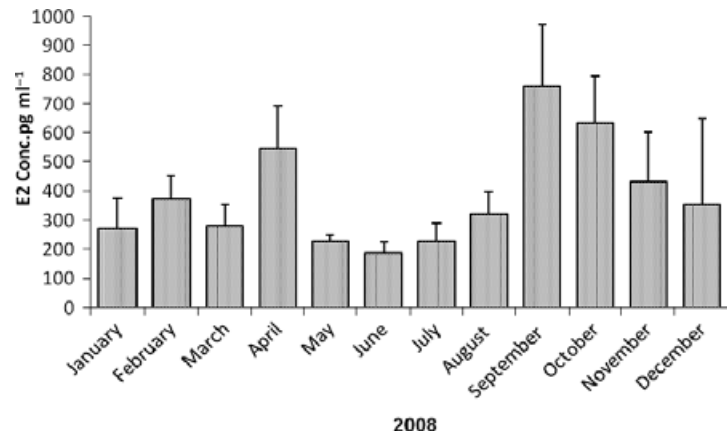


Figure 3 Seasonal variation in estradiol in female *Barbus altianalis*.

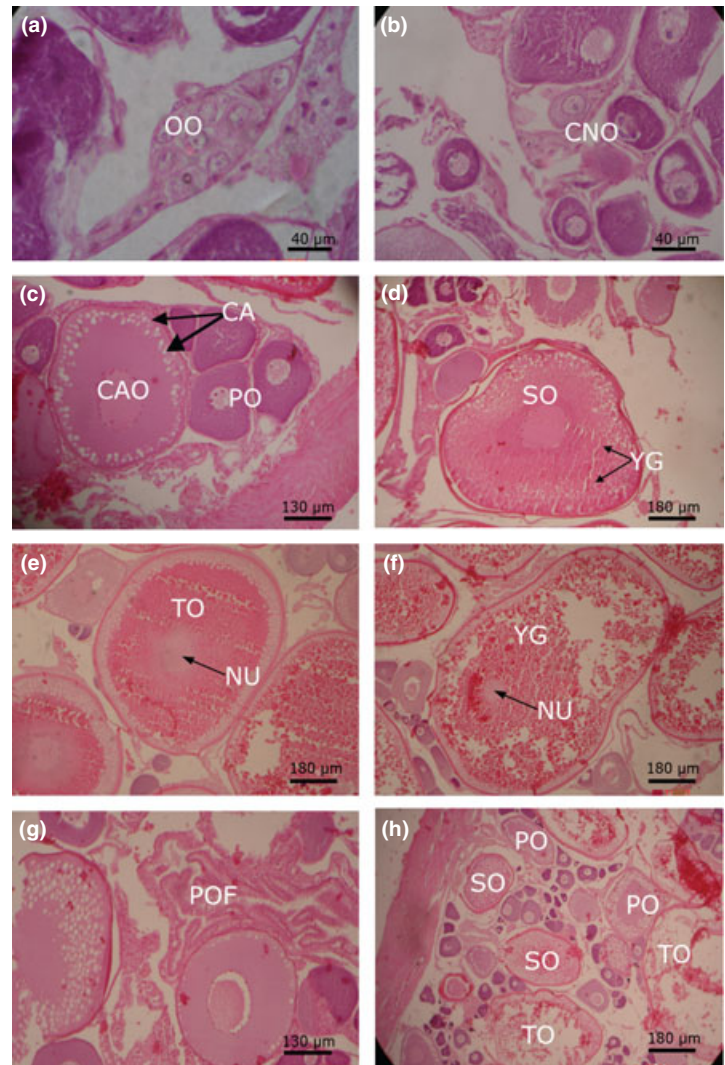


Figure 4 Histological sections of *Barbus altianalis* ovary showing oocyte maturation. Haematoxylin and Eosin (H & E) Stain. (a) Oogonia in nests (OO). (b) Chromatin nuclear oocyte (CNO). (c) Cortical alveoli oocyte (CAO) with cortical alveoli (CA) appearing at the periphery. PO are the perinucleolar oocytes. (d) Secondary yolk vesicle oocyte (SO) became more eosinophilic marking the increasing deposition of yolk globules (YG). (e) Eosinophilic tertiary yolk vesicle oocyte (TO) with the Nucleus (NU) still at the centre. (f) The yolk globules (YG) enlarge and the Nucleus (NU) moves to the periphery. (g) The post-ovulatory follicle (POF) remains in the ovary when the egg is expelled. (h) Groups of Oocytes at different stages of development can be seen in the ovary. This is typical of a batch spawner species.

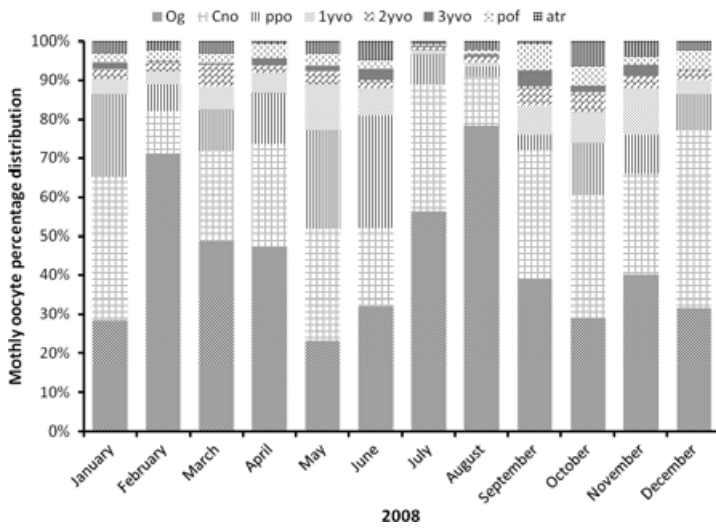


Figure 5 Annual oocyte distribution in *Barbus altianalis*.

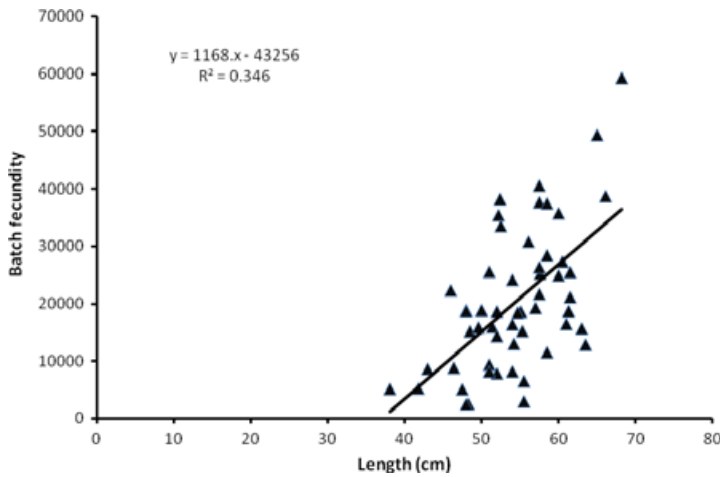


Figure 6 Fecundity relationships with length.

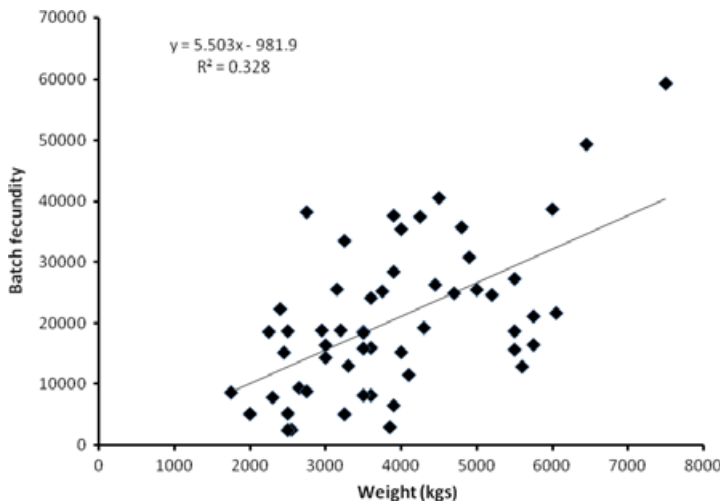


Figure 7 Fecundity relationship with eviscerated body weight.

Incubation and hatching

Ovulation was induced in 10 females by water current alone, whereas the other five fish that had not ovulated yet were injected with DAGIN and ovulated 12 h post injection. Mean fertilization rate was 55% ($P < 0.05$) in DAGIN induced fish and 72% ($P < 0.05$) in the water current induced fish. All the eggs were non-sticky and were sinking, measuring 2.97 ± 0.1 mm at stripping. Fertilized eggs increased in size from 2.97 ± 0.1 to 3.08 ± 0.4 mm and become translucent after 10 h (Fig. 8). Cellular cleavages began 30 min after fertilization and a mass of blastomeres forming a morula were observed 3 h later (Fig. 8a, b). Early embryo was noticed 20 h post fertilization with an embryonic axis developing somites (Fig. 8c). This stage was followed by a tail-free

embryonic stage 10 h later in which the tail separated from the yolk (Fig. 8d). Twitching movements of the embryo were visible through the egg shell after 15–20 h of incubation. At the time of hatching, the embryo was clearly visible through the egg membrane and occupied 75% of the egg space. The movements continued at a rate of 30 ± 5 per minute till hatching. The first hatching was observed 45 h post fertilization with the last hatching recorded after 72 h at $27 \pm 1^\circ\text{C}$. Mean hatchability was 37.3%. The newly hatched larvae (Fig. 8) measured 10 ± 1.0 mm TL. The larvae had a well-developed and rounded yolk sac and a thin tail. The larvae lay at the bottom of the hatching container in a horizontal position and moved only when disturbed. The yolk sac was visible for 2–3 days post hatching (Fig. 8e, f). Average egg and sac-larval survival was 55% and 11%

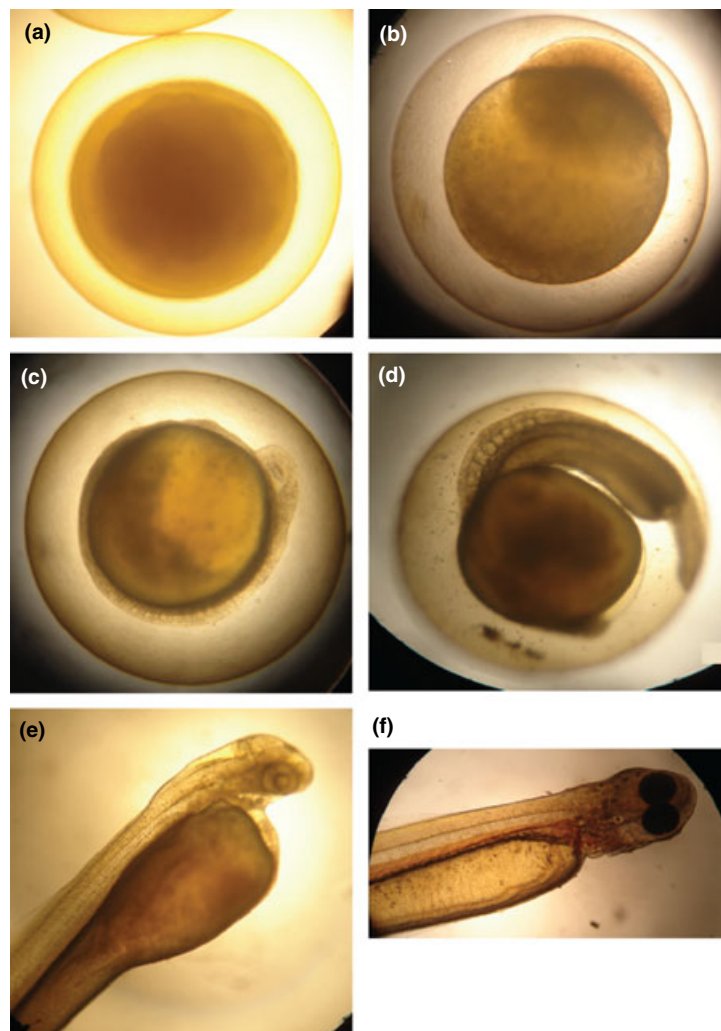


Figure 8 Embryonic developments in *Barbus altianalis* $\times 40$. (a) Early egg cleavage occurred 30 min at fertilization. (b) A morula forms 3 h after fertilization. (c) Early embryo formation at 20 h post fertilization. (d) Tail-free late embryonic stage at 30 h post fertilization. (e) Hatched larva after 45 h post fertilization with a reducing yolk sac $\times 1$. (f) 2–3 days after hatching, the yolk has been utilized and the trunk flattened.

respectively. The newly hatched larvae became active after the absorption of the yolk and swarm up to feed. They fed on fortified dry feed of crude protein 55% made by Raanan feed company (Akko, Israel).

Discussion

Information pertaining to patterns of gonadal recrudescence, spawning seasonality, synchronization of ovulation and fecundity is important for regulating reproduction of new farmed fish species. These data are, however, only available for a small number of teleost species of commercial importance (Tyler & Sumpter 1996) and largely absent for East African cyprinids. As a result, only a few East African cyprinids have been induced to spawn. It is widely acknowledged that the inability to obtain adequate seed supply is a major constraint to the initiation or expansion of aquaculture (Bromage 1995).

Although there has been a global increase in the number of farmed fish species since 1950 (Food & Agriculture Organization (FAO) 2006), very few species indigenous to Africa have been spawned and cultured possibly because the reproductive processes leading to spawning in natural environment has not been extensively studied in a considerable number of species.

It is known that the process leading to spawning in fish is controlled by endogenous biological rhythms as well as by environmental cues. Teleosts have inherent mechanisms for correlating gonadal structure and function with environmental factors that trigger spawning. This strategy allows for spawning when conditions are optimal for survival and growth of the progeny (Bye 1984; Munro 1990; Bromage, Porter & Randall 2001). The correlation between reproduction and the environment is mediated through the neuro-endocrine system, which perceives environmental cues and transduces signals that influence gonadal structure and their function (Peter & Yu 1997). Under natural conditions, environmental stimuli are detected and relayed to the brain resulting in a release of hormones and neurotransmitters, such as GnRH and dopamine, that regulate oogenesis and ovulation through their effect on gonadotropin production and release (Yaron & Levavi-Zermonsky 1986; Yaron 1995; Peter & Yu 1997; Levavi-Sivan, Bogerd, Mananos, Gomez & Lareyre 2010). Understanding of the hypothalamus–pituitary–gonadal

axis has enabled the use of various combinations of GnRH and dopamine antagonists to induce ovulation in several cyprinids including *Carassius auratus* (Sokolowska, Peter, Nahorniak, Pan, Chang, Crime & Weil 1984), *L. rohita*, *Cirrhinus mrigala* (Halder, Sen, Bhattacharya, Ray, Ghosh & Jhingran 1991), *C. carpio* (Drori *et al.* 1994) and *L. victorianus* (Rutaisire & Booth 2004). In this study, both water current and exogenous hormonal administration were found to be effective inducers of spawning in *B. altianalis* as could be deciphered from the fish that were found running without any hormonal administration and the ones that ovulated after injection with DAGIN.

Manipulation of environmental factors has been used in a number of teleosts to induce final oocyte maturation, ovulation and spawning (Mañanós, Zanuy & Carrillo 1997; Bromage *et al.* 2001; Kissil, Lupatsch, Elizur & Zohar 2001; Ito1, Yamasaki, Yamaguchi, Nagae & Soyano 2003; Kamanga, Kaunda, Mtimuni1, Maluwa & Mfitilodze 2004). Such knowledge of the reproductive responses of fish to specific environmental cues offers means by which the timing of spawning can be adjusted to provide fry and fingerlings on demand.

Fecundity studies found that *B. altianalis* had low level of relative fecundity (6 eggs g^{-1}) as compared with other cyprinids such as *L. victorianus* with 299 eggs g^{-1} (Rutaisire & Booth 2005), *C. carpio* with 123 g^{-1} (Bishai, Ishak & Labib 1975) and *Labeo horie* with 60–290 eggs g^{-1} of body weight (Dadebo, Ahlgren & Ahlgren 2003). The low fecundity could be attributed to the large size of eggs and the histological finding that the oocytes develop in batches. This was further confirmed by the few eggs released upon stripping (1500–3000 eggs kg^{-1} body weight).

Gonadosomatic index and the annual profile of E_2 showed no major variations, although there were slight peaks in April and September corresponding to the onset of rainfall. This is attributed to the asynchronous development of oocyte with vitellogenic oocytes present throughout the year. The slight peaks in GSI and E_2 during April and September and the occurrence of the highest number of post-ovulatory follicles during these months suggest that spawning in *B. altianalis* occurs throughout the year, but were more pronounced during the onset of rainfall. The onset of rainfall would cause a general change in water current, which was found in this study to stimulate

ovulations in addition to conferring survival advantages to the offspring through increased food availability as observed by Hails and Abdullah (1982) and Dadebo *et al.* (2003). Although *B. altianalis* can be easily induced to spawn by manipulating the water current, the few eggs released after stripping do not favour the stripping method, thus necessitating a method that allows for natural spawning and fertilization with regular collection of larvae. Such a method should include control of fungi infestation, which was a major challenge during the more than 45 h of egg incubation in this study.

The large size of the egg (2.97 ± 0.1 mm) and the corresponding size of hatchlings (10 ± 1 mm) confer an advantage to culture of *B. altianalis* as the larvae is large enough at the time of after absorption of yolk sac to take on large size particles such as the commercially available artemia or large zooplankton. The production of good quality seed of *B. altianalis* will require reducing observed egg and fry mortalities through optimization of artificial spawning condition, and defining the weaning protocol for raising the fry and fingerlings. From a socioeconomic point of view, successful induction of spawning by water current will be affordable to farmers in East Africa and should provide an impetus for culture of the species in the region.

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